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Quinone-induced protein handling changes: Implications for major protein handling systems in quinone-mediated toxicity

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ABSTRACT

Para-quinones such as 1,4-Benzoquinone (BQ) and menadione (MD) and ortho-quinones including the oxidation products of catecholamines, are derived from xenobiotics as well as endogenous molecules. The effects of quinones on major protein handling systems in cells; the 20/26S proteasome, the ER stress response, autophagy, chaperone proteins and aggresome formation, have not been investigated in a systematic manner. Both BQ and aminochrome (AC) inhibited proteasomal activity and activated the ER stress response and autophagy in rat dopaminergic N27 cells. AC also induced aggresome formation while MD had little effect on any protein handling systems in N27 cells. The effect of NQO1 on quinone induced protein handling changes and toxicity was examined using N27 cells stably transfected with NQO1 to generate an isogenic NQO1-overexpressing line. NQO1 protected against BQ–induced apoptosis but led to a potentiation of AC- and MD-induced apoptosis. Modulation of quinone-induced apoptosis in N27 and NQO1-overexpressing cells correlated only with changes in the ER stress response and not with changes in other protein handling systems. These data suggested that NQO1 modulated the ER stress response to potentiate toxicity of AC and MD, but protected against BQ toxicity. We further demonstrated that NQO1 mediated reduction to unstable hydroquinones and subsequent redox cycling was important for the activation of the ER stress response and toxicity for both AC and MD. In summary, our data demonstrate that quinone-specific changes in protein handling are evident in N27 cells and the induction of the ER stress response is associated with quinone-mediated toxicity.

Introduction

Quinones can undergo one-electron enzymatic reduction by NADPH-cytochrome P450 and NADH-cytochrome b5 reductases to generate semiquinone radicals that can redox cycle leading to the formation of reactive oxygen species (ROS) including superoxide anion radicals, hydrogen peroxide, and hydroxyl radicals. Quinones are also electrophiles and cellular damage due to these species can result from reaction with nucleophilic amino groups on proteins or DNA. In addition, covalent binding of quinones with cellular thiols, such as GSH or cysteine residues on proteins may lead to depletion of cellular GSH levels and/or protein arylation. Previous studies have attributed quinone-induced toxicity to two major mechanisms: (1) ROS formation and (2) arylation with macromolecule targets including histone, tubulin, topoisomerase II, and DNA (Gurbani et al., 2012; Monks and Jones, 2002; Ross, 2000). Few studies have reported quinone-induced changes in major protein handling systems, which include the 20/26S proteasome, the ER stress response, autophagy, and aggresome formation. 1,4-Benzoquinone (BQ) was previously shown to induce the ER stress response (Wang et al., 2006) but whether this resulted from ROS production or arylation and the relationship to other protein handling changes was not determined.

Aminochrome (AC) is a reactive ortho-quinone metabolite derived from dopamine and has been used as a model neurotoxin to study Parkinson's disease in cell and animal models (Segura-Aguilar et al., 2014). The mechanism of AC induced neurotoxicity is manifested via one-electron redox cycling leading to oxidative stress and/or arylation with cellular nucleophiles, such as GSH and alpha-synuclein (Conway et al., 2001; Norris, 2005). Both AC and BQ have been shown to disrupt the microtubule network and the mechanism was implicated as formation of covalent adducts with reactive-SH groups of tubulin and subsequent perturbation of microtubule assembly (Das et al., 2010; Paris et al., 2010; Santa-María et al., 2005). This evidence suggested an important role for arylation in quinone-induced protein handling changes. More importantly, data from our own work and others has shown that AC could exert an inhibitory effect on proteasomal activity (Jinsmaa et al., 2011; Zafar, 2006; Zhou et al., 2010), and AC was able to induce a significant increase in autophagosome formation (Gurbani et al., 2012; Monks and Jones, 2002; Muñoz et al., 2012; Ross, 2000). Our

Abbreviations: UPS, ubiquitin–proteasome system; HSPs, heat shock proteins; UPR, unfolded protein response; eIF2α, eukaryotic translation initiation factor 2 alpha; LC3, autophagy related microtubule-associated protein 1 light chain 3; NAD(P)H, quinone oxidoreductase 1 (NQO1); BQ, 1,4-Benzoquinone; MD, menadione; AC, aminochrome.

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previous work has also indicated a key role for the proteasome in the regulation of compensatory protein handling systems and the potential importance of proteasomal inhibition in the biogenesis of protein aggregates associated with Parkinson's disease (Xiong et al., 2013).

NAD(P)H:quinone oxidoreductase 1 (NQO1, DT-diaphorase) is generally considered to play a protective role in quinone-induced toxicity because it catalyzes two-electron reduction of quinones to less reactive hydroquinones thus decreasing the concentration of quinones available for one-electron reduction to semiquinones and arylation reactions (Ross, 2004; Ross and Siegel, 2004). However, hydroquinones generated by NQO1 may also be unstable to oxygen and can undergo autoxidation to produce ROS leading to toxicity (Paris et al., 2007; Ross, 2004; Watanabe et al., 2004).

Although the mechanisms of quinone toxicity have been extensively studied, the role of quinones in modulation of protein handling systems has not been investigated in detail. It is possible that redox active and electrophilic quinones may target the proteasome and result in the downstream modulation of other protein handling systems that could markedly amplify the consequences of a single quinone-induced arylation or oxidative event. In addition, quinones may have direct effects on other protein handling systems independent of any proteasomal effects. Proteasomal inhibition has been shown to perturb downstream compensatory protein handling changes (Choy et al., 2011; Xiong et al., 2013). We performed this study to determine whether quinones induce changes in major protein handling systems either independently or downstream of proteasomal inhibition and to define the role of NQO1 in modulating these changes.

Materials and methods

Materials and antibodies. 1,4-Benzoquinone (BQ), menadione (MD), duroquinone (DQ), 2,3-dimethoxy-1,4-naphthoquinone (DMNQ), NADH, dopamine hydrochloride, ATP disodium salt, digitonin, phenylmethylsulfonyl fluoride (PMSF), tunicamycin, mouse monoclonal anti-β-actin (A5441) and rabbit polyclonal anti-ubiquitin (U5379) antibodies were obtained from Sigma Chemical (St. Louis, MO, USA). Polyphenol oxidase (mushroom tyrosinase, LS003793) was purchased from Worthington Biochemical (Lakewood, NJ, USA). Dithiothreitol was purchased from Fisher Scientific (Pittsburgh, PA, USA). MTT [Thiazolyl blue] was obtained from Research Products International (Mount Prospect, IL, USA). MG132, purified human 20S proteasome (BML-PW8720), mouse monoclonal anti-Hsp27, anti-Hsp70 antibodies and rabbit polyclonal anti-Hsp90α antibody were obtained from Enzo (Farmingdale, NY, USA). FITC/ annexin V (640906) and annexin V binding buffer (422201) were purchased from BioLegend (San Diego, CA, USA). The fluorescently labeled proteasome substrate Suc-Leu-Leu-Val-Tyr-AMC was purchased from Bachem (Torrance, CA, USA). Rabbit polyclonal anti-LC3 (NB100-2331) was obtained Novus (Littleton, CO, USA). Mouse monoclonal anti-NQO1 (A180) antibody, rabbit polyclonal anti-phospho-eIF2α (Ser51, #9721), anti-eIF2α (#9722) and anti-caspase-3 (#9662) antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Rabbit polyclonal anti-caspase 12 (ab62484) and anti-NQO1 (ab34173) antibodies were obtained from Abcam (Cambridge, MA, USA).

Cell culture and treatment. N27 rat mesencephalic dopaminergic cells were obtained from Dr. Curt Freed, Department of Clinical Pharmacology and Toxicology, University of Colorado Anschutz Medical Campus, Aurora CO (Adams et al., 1996). N27 cells were cultured in RPMI-1640 medium (Cellgro, Manassas, VA, USA) containing 10% (v/v) fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin (Cellgro, Manassas, VA, USA) in a humidified atmosphere of 5% CO_2 at 37 °C. Stock solutions of BQ and MD (10 mM) were prepared in ethanol and AC (1 mM) was generated as described below. All stock solutions were used immediately after preparation.

Stable transfection of N27 cells with human wild-type (wt) hNQO1. Stable transfection of N27 cells was carried out as described previously (Zafar et al., 2006). Briefly, the CMV-driven mammalian expression vector pcDNA3 containing human NQO1*1 wild type cDNA was transfected by electroporation into N27 cells with neomycin as the selection marker. Clones were selected after 7 to 10 days and examined for both NQO1 enzymatic activity and NQO1 immunoreactivity. The NQO1-transfected (clone 4) cells are routinely examined for NQO1 expression by activity assay, immunoblot analysis and confocal microscopy.

Measurement of NQO1 activity. NQO1 activity was determined spectrophotometrically in cell lysates using the dicumarol-inhibitable reduction of 2,6-dichlorophenol-indophenol (DCPIP) at 600 nm as described previously (Benson et al., 1980). Protein concentrations were determined using the method of Lowry (Lowry et al., 1951).

Aminochrome preparation. Aminochrome was prepared by enzymatic reaction (300 μl, room temperature) containing 3.3 mM dopamine and 500 μg tyrosinase in 25 mM Tris–HCl buffer (pH 7.4). After 3 min at room temperature the reaction mixture was centrifuged (13,000 rpm for 5 min at 4 °C) through a 100-kDa molecular mass cutoff membrane filter (Millipore Corporation, Bedford, MA) to remove tyrosinase and prevent additional tyrosinase-catalyzed oxidative reactions. After centrifugation the filtrates were collected and stored on ice. To determine the concentration of aminochrome generated 5 μl of the filtrate was added to 995 μl of 25 mM Tris–HCl buffer (pH 7.4) and the absorption was determined at 474 nm. The concentration of aminochrome was calculated using a molar extinction coefficient of 3058 at 474 nm (Baez et al., 1997).

MTT growth inhibition assay. Quinone toxicity was initially determined using the MTT assay and confirmed using trypan blue and apoptosis/necrosis assays (below). For MTT assay, N27 cells were seeded at 2000 cells per well in 96-well plates (in triplicate). After 24 h the cells were treated with BQ or MD in complete medium or with AC in serum-free medium for 24 h. After drug treatments, the medium were removed and replaced with MTT (1 mg/ml) containing complete medium. After 4 h the MTT containing medium was removed and the MTT formazan product was extracted from cells with dimethyl sulfoxide. The optical density of the extract was determined at 550 nm with a microplate reader. IC_{50} values were defined as the concentration of quinone that resulted in a 50% reduction in cell density compared to untreated controls.

Trypan blue exclusion assay. Cells were seeded at 1.5×10^5 cells into 60 mm × 15 mm tissue culture dishes in 3 ml of complete medium and allowed to attach and grow for 2 days. Following treatment with quinone for the indicated times, cell pellets containing both floating and attached cells were collected and then resuspended in 1 ml PBS. To assess cell viability the cell suspension (10 μl) was stained with 10 μl trypan blue dye (0.4% w/v in PBS). The number of trypan blue positive cells was determined using a Life Technologies Countess™ automated cell counter immediately after staining.

Apoptosis assay. Apoptosis was determined by flow cytometry using FITC-conjugated anti-annexin V and propidium iodide (PI) as previously described (Benz et al., 2006; Xiong et al., 2013). After 24 h treatment with quinones, cell pellets including both the floating and attached cells were collected and gently resuspended in 500 μl of annexin V binding buffer and incubated with anti-annexin V antibody (2 μl) and PI (0.7 μl of 100 μg/ml stock) for 10 min at 37 °C in the dark. Samples were kept on ice, and then analyzed using a BD Biosciences FACS Calibur Flow Cytometer (San Jose, CA, USA). The fluorescence was measured at 530 nm (FL1, FITC) and PI at above 600 nm (FL2), and the data was acquired and analyzed using Cellquest software (Becton-Dickenson, Mountainview, CA, USA).